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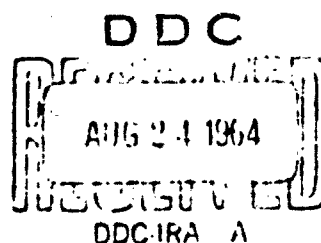
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TECHNICAL MANUSCRIPT 140

PHYSICOCHEMICAL PROPERTIES OF PURIFIED STAPHYLOCOCCAL ENTEROTOXIN B

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PHYSICOCHEMICAL PROPERTIES OF PURIFIED STAPHYLOCOCCAL ENTEROTOXIN B

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ABSTRACT

Staphylococcal enterotoxin B exhibited a high degree of molecular homogeneity as determined by synthetic boundary spreading, approach-to-equilibrium sedimentation, and sedimentation equilibrium distribution in a density gradient. Its partial specific volume ($0.743 \text{ cm}^3/\text{gm}$) and infrared spectral absorption were typical of simple proteins. The molecular weight by sedimentation-diffusion was found to be 35,300 and was in good agreement with results by the Archibald procedure. There was stability in sedimentation behavior over a wide pH range (5 to 10) and an observed transition to a more extended structural form at pH 3.8. A net hydration of 0.075 gram of water per gram of protein was evaluated by extrapolation to zero sedimentation rate in aqueous sucrose, and values of 0.158 and 0.136 gram per gram were obtained from measurements of enterotoxin density (1.286 gm/cm^3) and solvated molecular weight (40,100), respectively, in buoyant cesium chloride solution. Intrinsic viscosity and sedimentation-diffusion data were combined to yield a value of 2.14×10^6 for the Scheraga-Mandelkern parameter β . The latter is discussed in terms of its implications as to the nature of the hydrodynamic enterotoxin unit.

I. INTRODUCTION

Staphylococcal enterotoxin B has been prepared in a highly purified state by Schantz and co-workers,¹ primarily by a column chromatographic procedure.

This report deals with the results of an investigation of a number of molecular parameters of the purified enterotoxin, including its homogeneity, molecular size, shape, and hydration, based principally on studies of ultracentrifugal sedimentation, diffusion, buoyant behavior, and viscosity.

II. MATERIALS AND METHODS

A. ENTEROTOXIN SAMPLES

Enterotoxin was purified in several batches from cultures of Staphylococcus aureus.¹ After it was determined that the different batches were indistinguishable in biological activity (the dose, ED₅₀, to produce emesis or diarrhea in rhesus monkeys was 0.1 to 0.3 microgram per kg of animal weight), sedimentation behavior, and other properties, these were pooled, dialyzed to eliminate nearly all salts, and lyophilized. For physical measurements, samples from this stock of dried material were dissolved in appropriate aqueous buffers, which in most cases was 0.05 M potassium phosphate, pH 6.8. Solutions of enterotoxin were also initially dialyzed against the buffer being used as solvent when this was considered necessary, such as in the synthetic boundary and approach-to-equilibrium sedimentation procedures.

B. ULTRACENTRIFUGAL ANALYSIS

Most of the data were obtained by ultracentrifugation, using a Model E Spinco instrument equipped with a rotor temperature-indicating and -control unit. Both the conventional 12-mm cell with four-degree sector and the valve-type synthetic boundary cell were used in sedimentation velocity experiments. A valve-type synthetic boundary cell was also used in measuring diffusion coefficients. In determining molecular weight during the approach to equilibrium by the method suggested by Archibald,² the procedure followed was essentially that proposed by Klainer and Kegeles.³ This required use of both conventional and synthetic boundary cells. Finally, in determining molecular weight and buoyant density by isopycnic sedimentation equilibrium, a double-sector capillary-type synthetic boundary cell was used as suggested by Ifft and Vinograd.⁴

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C. ADDITIONAL PHYSICAL STUDIES

Other physical studies included determination of partial specific volume by pycnometry with a Lipkin graduated dual-limb pycnometer (Ace Glass Company), measurement of intrinsic viscosity with a calibrated Cannon-Fenske capillary viscometer, and infrared spectral absorption in a Perkin-Elmer Model 21 spectrophotometer.

D. MEASUREMENTS AND CALCULATIONS

All of the ultracentrifugal analyses were carried out with schlieren optics. Measurements of photographic plates were made either directly with a comparator or from enlarged tracings. The analysis of sedimenting boundaries to determine diffusion coefficients and polydispersity involved measurement of the second moments of schlieren curves. The integrals required for these, as well as for the measurements of c_m and c_b in the Archibald method, and of buoyant density by isopycnic sedimentation equilibrium, were obtained by numerical integration using Gauss's mechanical quadrature formula with the roots and weight coefficients determined by Lowan, Davids, and Levenson.⁵ This method requires about half the number of ordinate measurements and calculations, for a particular degree of accuracy, as compared with the usual methods of numerical integration.

III. EXPERIMENTS

A. SEDIMENTATION VELOCITY

Through velocity ultracentrifugation alone, it was possible to determine a range of molecular properties of the enterotoxin in solution that included heterogeneity, sedimentation and diffusion coefficients (which, together with a partial specific volume measurement, yielded values for molecular weight and frictional ratio), particle stability with changing pH, and net particle hydration in buoyant sucrose solution. Early experiments were carried out in a conventional ultracentrifuge cell, but in all cases, the use of a valve-type synthetic boundary cell was found to be advantageous. This procedure shortened the duration of centrifuge runs and simplified subsequent calculations.

Representative schlieren curves obtained in synthetic boundary runs with solutions of enterotoxin in 0.03 M potassium phosphate buffer, pH 6.8, are shown in Figure 1. The areas under the refractive gradient curves, which in all cases revealed only a single, symmetrical sedimenting boundary, were

found, after correction for radial dilution, to be independent of either centrifugal field strength or time during cell traversal. Although this indicated no apparent heterogeneity in the solutions of enterotoxin, several more sensitive tests of homogeneity were also performed and will be described subsequently.

Sedimentation coefficients, s_{20}^w , were obtained from the movement of the maximum ordinate in the schlieren curves and are listed in Table I for several initial concentrations of enterotoxin. The concentration dependence of s_{20}^w was low, for the measured value with a solution containing one gram per 100 milliliters was less than seven per cent below the value 2.89 S obtained by linear extrapolation to infinite dilution.

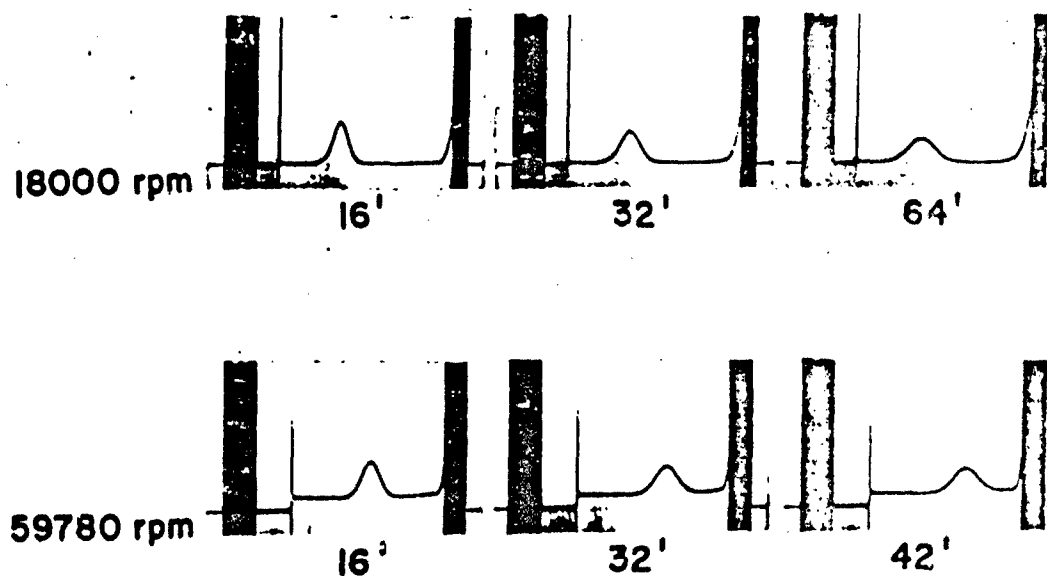


Figure 1. Synthetic Boundary Sedimentation Diagrams of Staphylococcal Enterotoxin.

One gram per 100 ml in 0.05 M phosphate buffer, pH 6.8, at two different rotor speeds. Time after reaching full speed is indicated below diagrams. Temperature, 20°C; schlieren angle, 80 degrees.

TABLE I. SEDIMENTATION, DIFFUSION, AND MOLECULAR WEIGHT OF STAPHYLOCOCCAL ENTEROTOXIN BY VELOCITY ULTRACENTRIFUGATION^{a/}

| Sedimentation | | Diffusion | | Molecular Weight |
|-----------------|--------------------|-----------------|---|----------------------|
| Conc, gm/100 ml | s_{20}^w , s | Conc, gm/100 ml | D_{20}^w , (cm ² /sec)x10 ⁷ | |
| 1.00 | 2.69 | 1.00 | 7.04 | 35,300 ^{c/} |
| 0.48 | 2.83 | 0.547 | 7.33 | |
| 0.29 | 2.80 | 0.181 | 7.61 | |
| 0.11 | 2.88 | 0 | 7.72 ^{b/} | |
| 0 | 2.89 ^{b/} | | | |

a. In 0.05 M phosphate buffer, pH 6.8, at 20°C.

b. By linear extrapolation.

c. Using the extrapolated values for s_{20}^w and D_{20}^w , and a measured value for V_{20} of 0.743.

The sedimentation velocity data were also analyzed for boundary spreading as a further test of homogeneity. This was done according to the treatment of Baldwin and Williams,⁶ in which the effects of concentration and pressure on s are neglected, and the boundary spreading, expressed in terms of the second moment or square of the average deviation about the mean, σ^2 , in the gradient sedimentation curves, is assumed to be caused by heterogeneity and diffusion, i.e.,

$$\sigma^2 = (pw^2rt)^2 (1 + \dots) + 2Dt/1-\omega^2st \quad (1)$$

In this relation, ω = angular velocity, r = distance of maximum ordinate from the axis of rotation, t = time, and p and D are polydispersity and diffusion coefficients, respectively. For a homogeneous material, the first term in Equation (1) drops out and a plot of $\sigma^2 (1-\omega^2st)$ against t should be linear and should yield an apparent value for D (from one half the slope). Figure 2 shows an application of this boundary spreading analysis with data from a pair of synthetic boundary runs with solutions of enterotoxin (1 gm/100 ml) at centrifugal fields of about 267,000g and 23,500g, respectively. Both sets of data show a linearity in the plots of $\sigma^2(1-\omega^2st)$ against t with least square slopes that are nearly equal and that yield values for the apparent diffusion coefficient D_{20}^w of 6.97×10^{-7} cm² per second and 7.04×10^{-7} cm² per second. Inasmuch as the apparent values for

D are nearly independent of centrifugal field strength, it may be concluded that the boundary sharpening effects due to the concentration and pressure dependence of s are negligible. These experiments, therefore, not only indicate a high degree of enterotoxin homogeneity, but also yield apparent values for D that should not be significantly different from true values.

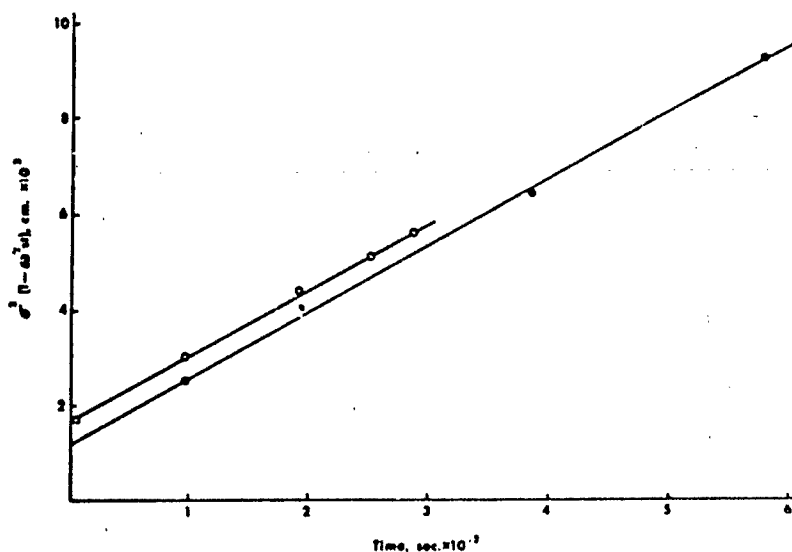


Figure 2. Analysis of Boundary Spreading for Staphylococcal Enterotoxin.

The rate of increase of the field-corrected second moment, $\sigma^2(1-w^2)st$, is plotted from data obtained in synthetic boundary experiments with solutions containing one gram enterotoxin per 100 ml at centrifugal fields about 267,000g (O) and 23,500g (●), respectively.

Values for D_{20}^W , calculated as outlined above from the rate of increase of the second moment about the mean in the gradient curves from synthetic boundary experiments at 18,000 rpm with solutions of enterotoxin at different initial concentrations, are listed in Table I. These extrapolate linearly to a value of $7.72 \times 10^{-7} \text{ cm}^2$ per second at zero concentration. A measurement of the partial specific volume V_{20} of enterotoxin in water, using a 25-ml pycnometer, yielded a value of 0.743. The molecular weight of the enterotoxin, as determined by combining V_{20} with s_{20}^W and D_{20}^W at infinite dilution in the Svedberg equation, is 35,300. These data also yield a value of 1.264 for the frictional ratio, f/f_0 .

The results of a study of the effect of solvent pH on the sedimentation behavior of the enterotoxin are given in Table II. Over the pH range 5 to 10, the gradient curves were indistinguishable from those shown in Figure 1 and showed no significant change in s_{20}^w . At pH 3.8, however, both the boundary spreading (and hence diffusion) rate and s_{20}^w were considerably reduced, indicating a structural transition to a more solvated or more extended molecular configuration with no apparent change in molecular weight.

TABLE II. VARIATION OF SEDIMENTATION COEFFICIENT WITH pH STAPHYLOCOCCAL ENTEROTOXIN, ONE GRAM PER ONE HUNDRED MILLILITERS, IN 0.1 IONIC STRENGTH BUFFERS

| Buffer | pH | s_{20}^w S |
|-----------|------|-----------------|
| Carbonate | 10.0 | 2.74 |
| Borate | 8.6 | 2.76 |
| Phosphate | 6.8 | 2.69 |
| Acetate | 5.0 | 2.71 |
| Acetate | 3.8 | 2.34 |

B. APPROACH TO EQUILIBRIUM

This procedure, wherein the ultracentrifuge is employed "as a computing machine to solve its own equation" was used for direct measurements of the molecular weight of enterotoxin. Experiments were carried out at initial enterotoxin concentrations of 1.0 and 0.5 gram per 100 ml in 0.05 M phosphate buffer, pH 5.8, at 20°C and a rotor speed of 18,000 rpm. Values of the molecular weight at the meniscus, M_m , and at the bottom, M_b , of the cell were calculated from measurements of the schlieren curves (Figure 3) recorded at various time intervals and are listed in Table III.

A comparison of the various values of molecular weight provides a further test for the homogeneity of the enterotoxin. Neither the comparison of M_m with M_b values, nor the variation of either of these with time, provides any evidence of polydispersity, in agreement with the conclusions from velocity sedimentation analyses. While a higher internal precision would have been desirable, the prevailing trend in the values for apparent molecular weight may be partially explained on the basis of concentration dependence. The average (35,100) of the values obtained at an initial concentration of 0.5 gram per 100 ml is in good agreement with that determined by sedimentation velocity — diffusion measurement (35,300).

TABLE III. MOLECULAR WEIGHT OF STAPHYLOCOCCAL ENTEROTOXIN BY ARCHIBALD METHOD

| Conc, ^a / gram/100 ml | Time, ^b / min | M _m | M _b | M avg |
|-------------------------------------|-----------------------------|----------------|----------------|----------|
| 1.0 | 20 | 33,800 | 32,400 | |
| | 52 | 33,600 | 33,700 | |
| | 100 | 35,000 | 33,100 | |
| | | | | 33,600 |
| 0.5 | 16 | 33,700 | 32,600 | |
| | 32 | 35,700 | 34,900 | |
| | 64 | 36,300 | 37,200 | |
| | | | | 35,100 |

a. In 0.05 M potassium phosphate buffer, pH 6.8 at 20°C.

b. At rotor speed of 17,970 rpm.

C. BUOYANT BEHAVIOR OF ENTEROTOXIN IN THE ULTRACENTRIFUGE

Both velocity and equilibrium ultracentrifugation were employed to study the buoyant behavior of enterotoxin. These resulted in measurements of the buoyant density, net hydration, and apparent solvated molecular weight of the toxin particles and provided a further test of molecular homogeneity.

The velocity measurements were carried out with solutions of enterotoxin (1 gm/100 ml) in 0.05 M phosphate buffer, pH 6.8, containing varying amounts (0 to 40%) of sucrose. Use of a synthetic boundary cell prevented sucrose gradients from interfering with measurements of s of the enterotoxin. A plot of $\eta_r s$ versus ρ , where η_r and ρ are the solvent viscosity (relative to water) and solvent density, respectively, is shown in Figure 4. The data follow a straight-line plot with high precision and extrapolate to a value of 1.314 gram per cm³ for the compositional buoyant density ρ_0^0 or density at atmospheric pressure corresponding to zero sedimentation rate.

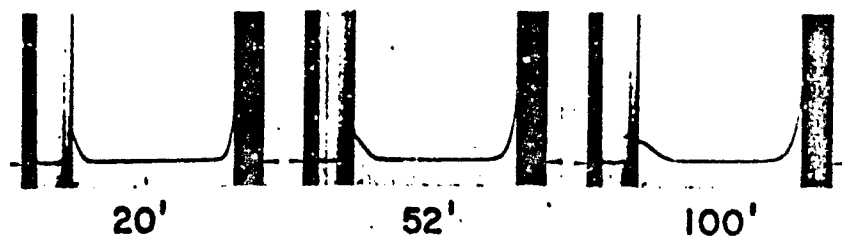


Figure 3. Ultracentrifuge Schlieren Patterns, During the Approach to Sedimentation Equilibrium, of Staphylococcal Enterotoxin.

One gram per 100 ml in 0.05 M phosphate buffer, pH 6.8, at 20°C. Time after reaching full speed (18,000 rpm) is indicated below each pattern. Schlieren diaphragm angle, 80 degrees.

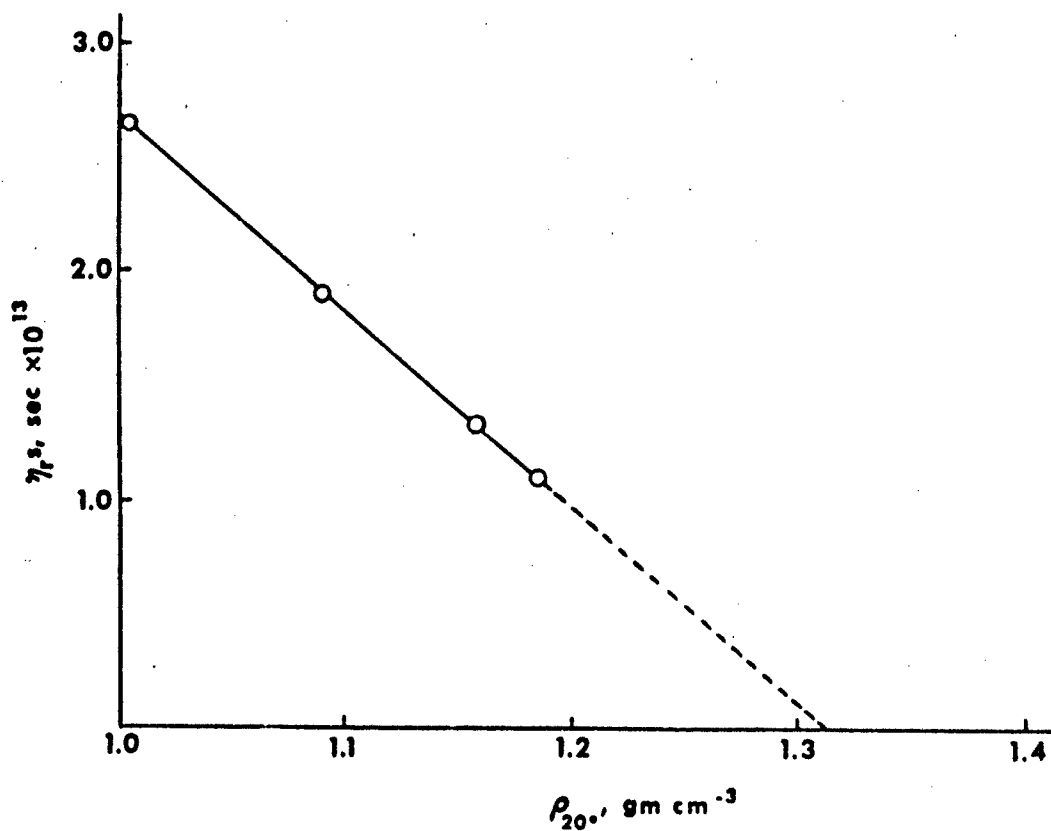


Figure 4. Sedimentation of Staphylococcal Enterotoxin as Function of Solvent Density.

One gram enterotoxin per 100 ml in 0.05 M potassium phosphate buffer, pH 6.8, containing varying amounts of sucrose.

In the equilibrium study, enterotoxin was banded in a cesium chloride gradient formed centrifugally at 56,100 rpm and 20°C. The correct baseline was determined by running the toxin-cesium chloride and reference cesium chloride solutions, respectively, in the two channels of a double-sector synthetic boundary cell. The refractive gradient curves of the toxin and the reference solutions were simultaneously recorded with the schlieren optical system. The comparatively low molecular weight of the enterotoxin resulted in broad banding at equilibrium, and it was therefore important that the initial cesium chloride concentration be carefully selected. In this connection, an exploratory experiment was carried out in which the cesium chloride concentration used was based upon the previously determined density (1.314 gm/cm³) of buoyant sucrose solution. This turned out to be too high, but the experiment was useful in that it yielded an estimate of the concentration of isopycnic cesium chloride solution. Consequently, the next run was performed with four milligrams per ml of enterotoxin dissolved in 2.58 molar cesium chloride. The duration was 26 hours, but an analysis of the schlieren photographs taken at successive time intervals showed that equilibrium had been reached after 20 hours.

A typical schlieren photograph of the enterotoxin at equilibrium in a cesium chloride density gradient is shown in Figure 5. The biphasic refractive gradient curve representing the enterotoxin in cesium chloride is superimposed on the simultaneously recorded baseline of the reference cesium chloride solution. It is of interest that the enterotoxin band is barely contained within the gradient column, which was calculated to have a compositional density range between 1.217 and 1.366 grams per cm³. Materials of lower molecular weight would require conditions that produce a steeper gradient, e.g., higher angular velocity or a denser salt.

The isopycnic radial distance r^0 was determined from the position of the mode or intersection of the biphasic gradient curve with the reference solution baseline. The index of refraction n^0 , at r^0 was evaluated from the initial reference solution refractive index n_0 and the reference solution baseline by the relation

$$n^0 = n_0 + \int_{r_m}^{r^0} \frac{\partial n}{\partial r} dr + \frac{1}{(r_b^2 - r_m^2)} \left[\int_{r_m}^{r_b} r^2 \frac{\partial n}{\partial r} dr - r_b^2 \int_{r_m}^{r_b} \frac{\partial n}{\partial r} dr \right] \quad (2)$$

which is based on the assumption that the total contents of the cell are independent of time.⁸ In Equation (2), r_m and r_b are the radial distances at the meniscus and bottom of the cell, respectively. Because of the nearly central position of r^0 on the gradient column, n^0 was nearly equal to n_0 , and corresponded to a compositional buoyant density ρ_0^0 of 1.286 grams per cm³.

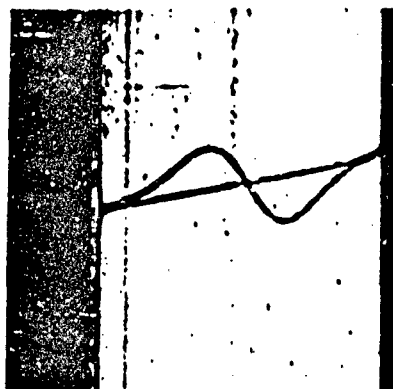


Figure 5. Equilibrium Distribution of Staphylococcal Enterotoxin in a Cesium Chloride Gradient at 20°C.

Simultaneously recorded schlieren patterns for a solution of four milligrams enterotoxin per ml in 2.58 molal cesium chloride and for a reference cesium chloride solution, respectively. Recorded 25 hours after reaching full speed, 56,100 rpm, at schlieren angle of 70 degrees.

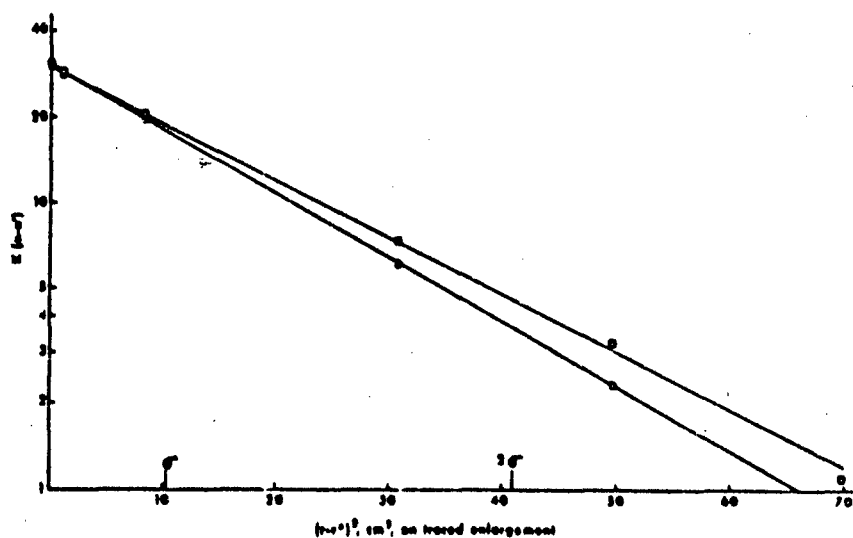


Figure 6. Analysis of the Distribution of Enterotoxin in a Cesium Chloride Density Gradient at Sedimentation Equilibrium in the Experiment Illustrated in Figure 5.

The top and bottom halves of the distribution are represented by \square and \circ , respectively. For a Gaussian distribution, the plot shown is linear with slope inversely proportional to σ^2 . Distances from r^0 corresponding to σ and 2σ are noted by arrows along the abscissa.

The net or selective hydration, h , was calculated from the relation

$$h = \frac{1 - \rho_o^o \bar{V}}{\rho_o^o \bar{V} - 1} \quad (3)$$

in which \bar{V}_w is the partial specific volume of water. Values of 0.075 and 0.158 gram of water per gram of enterotoxin in buoyant sucrose and cesium chloride, respectively, were thus obtained.

The equilibrium distribution of enterotoxin on the gradient column was determined from an enlarged tracing of the curves in Figure 5. Numerical integration of the two lobes of the enterotoxin gradient curve and reference solution baseline at various radial distances in the cell yielded values of $K(n-n')$ where n and n' are the refractive indices of the enterotoxin solution and baseline solution, respectively, and K is a machine constant depending on magnification factors and other optical parameters of the ultracentrifuge. As shown in Figure 6, a plot of $\ln K(n-n')$ against $(r-r^o)^2$ is linear for both halves of the gradient curves, indicating a Gaussian concentration distribution for the enterotoxin, and therefore both density and molecular weight homogeneity. However, the standard deviation σ obtained from the slope of the plot is somewhat higher at the top side (0.165 cm) of the band than at the bottom side (0.156 cm). A skewness of this nature was also observed for bovine serum mercaptalbumin by Ifft and Vinograd,⁴ who attributed this behavior to the non-constancy of the density gradient.

In order to calculate the solvated molecular weight M_s of the banded enterotoxin from the expression derived by Meselson, Stahl, and Vinograd,⁹ i.e.,

$$M_s = \frac{RT \rho^o}{\sigma^2 (d\rho/dr)^o w^2 r^o} \quad (4)$$

the only remaining quantities to be determined were ρ^o , the physical buoyant density, and $(d\rho/dr)^o$, the density gradient at r^o . A simple correction⁴ for the compressibility of the cesium chloride solution, at the pressure at r^o in the rotating liquid column, was used to derive ρ^o from ρ_o^o . Schlieren and refractive index measurements led to a value for $(d\rho/dr)^o$, making use of the relation

$$\frac{d\rho}{dr} = \frac{dn}{dr} \times \frac{d\rho}{dn} \quad (5)$$

Using an average of the values of σ for the top and bottom sides of the banded macromolecules, an apparent molecular weight of 40,100 is obtained for the solvated enterotoxin in buoyant cesium chloride. Table IV summarizes the equilibrium data.

TABLE IV. RESULTS OF EQUILIBRIUM DENSITY GRADIENT
EXPERIMENT WITH 4 mg/ml ENTEROTOXIN
IN CsCl SOLUTION AT 20°C

| | |
|--|--------|
| ρ_o^o , gram cm ⁻³ | 1.286 |
| ρ^o , gram cm ⁻³ | 1.293 |
| $(d\rho/dr)^o$, gram cm ⁻⁴ | 0.133 |
| σ_{top} , cm | 0.165 |
| σ_{bot} , cm | 0.156 |
| h , gram/gram protein | 0.158 |
| M_s | 40,100 |

D. OTHER MEASUREMENTS

All of the data¹ obtained in the course of purifying the enterotoxin indicated it to be a simple protein. The measurement for V_{20} (0.743) also supports this conclusion. An additional analysis was carried out by infrared spectrophotometry with a dried film of enterotoxin formed from a water solution on a KRS-3 disk. The spectral curve (Figure 7) contained the characteristic polypeptide absorption bands and closely approximated the curve for bovine serum albumin. Moreover, there was no indication of a band in the 9- to 10-micron interval such as was observed by Levi, Matheson, and Thatcher¹⁰ with crude extracts, and which they associated with both enterotoxic activity and the presence of a phospholipid moiety.

Measurements of reduced viscosity were made at 20°C in 0.05 M phosphate, pH 6.8, over the concentration range 0.3 to 1.7 grams per 100 ml. These yielded a value of 0.0392 (gm/100 ml)⁻¹ for the intrinsic viscosity $[\eta]$. By coupling this result with the previously obtained values for s_{20} , V_{20} , and M (2.89 S, 0.743, and 35,300, respectively) according to the treatment of Scheraga and Mandelkern,¹¹ a value of 2.14×10^6 is obtained for β , a parameter related to the axial ratio of the effective hydrodynamic ellipsoid.

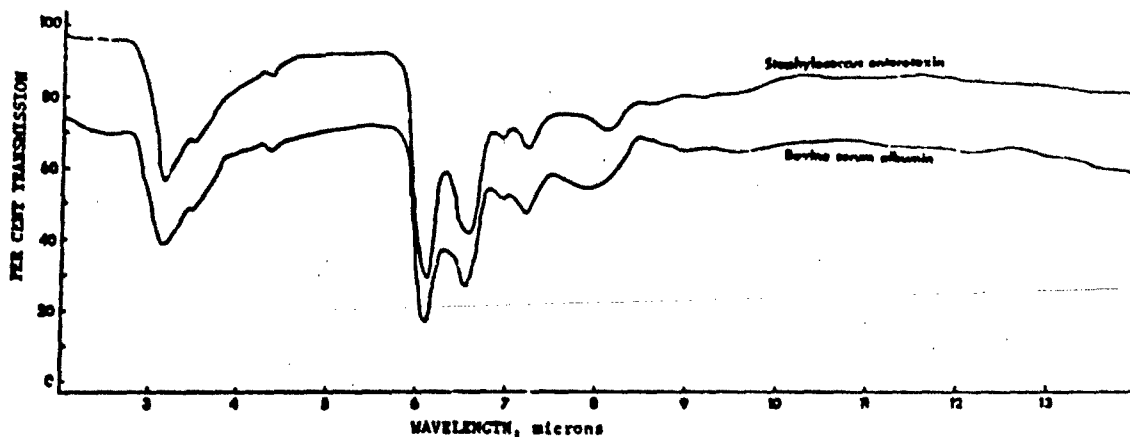


Figure 7. Infrared Absorption Spectra of Staphylococcal Enterotoxin and Bovine Serum Albumin.

The curve for albumin was "lowered" by a distance corresponding to 20 per cent transmission to facilitate comparison.

This value for β , by itself, does not rule out oblate ellipsoids of any axial ratio, but those with axial ratios higher than 5 appear to bear little resemblance to the sedimenting unit inasmuch as they would have effective hydrodynamic volumes V_e lower than that derived from the partial specific volume, a condition that is contradicted by the measurements of buoyant density. On the other hand, β is a much more sensitive indicator of axial ratio for a prolate ellipsoid; the value for enterotoxin corresponds to major and minor axes of 92 Å and 36 Å, respectively, and 1.23 cm³ per gram of protein for V_e .

IV. DISCUSSION

Several kinds of ultracentrifugal analyses, including velocity boundary spreading, approach-to-equilibrium sedimentation, and equilibrium distribution in a salt gradient, support the conclusion that the purified staphylococcal enterotoxin possesses a high degree of molecular homogeneity. The data presented also show it to be a reasonably compact protein molecule with a molecular weight slightly above 35,000. In addition to its stability over a wide pH range (from 5 to 10, at least) it has also been observed to be physically stable in solution for long periods of time at ambient temperatures (about 20°C). These characteristics of molecular homogeneity and stability appear to make it a useful standard material in its size range for physicochemical studies of proteins. The absence of dissociation over a broad range of pH also supports existing evidence from terminal residue analysis¹ that the enterotoxin consists of a single polypeptide chain.

Using the data of Robinson and Stokes¹² on osmotic coefficients at 25°C, the water activities of buoyant sucrose and cesium chloride solutions were calculated to be 0.868 and 0.922, respectively. Although this difference alone may account for the variance in the measured values of net hydration (0.075 gm/gm in sucrose and 0.158 gm/gm in cesium chloride), other factors such as solute-solute interaction cannot as yet be excluded. A value for net hydration in cesium chloride (0.136 gm/gm) can also be obtained from a comparison of the solvated and anhydrous molecular weights (40,100 and 35,300, respectively). It is not surprising that this is lower than the figure obtained from ρ_2 , for the value of $(d\rho/dr)$ used in Equation (4) is too large¹³ in that it neglects the change in the hydration of the protein through the band and therefore leads to apparent values of M_2 that are too small.

It is of interest to speculate on the meaning of the difference between the effective specific and partial specific volumes (1.23 and 0.743, respectively), although no basis exists at this time for any definite conclusions. One possibility is the inclusion of solvent in the interior of a somewhat swollen protein molecule. On the other hand, if the prolate ellipsoidal model is a reasonable approximation of the hydrodynamic unit, the volume difference may be due entirely to an outer shell of bound solvent 3.7 Å (or about a monomolecular layer) thick surrounding a tightly folded polypeptide chain. Other studies, such as low-angle X-ray scattering, may provide a firmer basis for an attempt such as this to relate the values of V and V_e .

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